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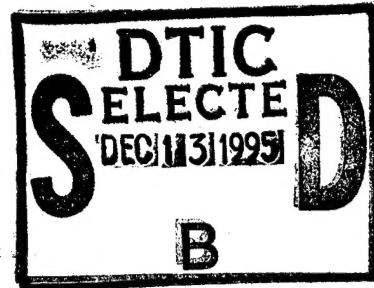
TITLE: Modulation of Cyclin Expression by C-MYC in Malignant and Nonmalignant Mammary Epithelial Cells

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INTRODUCTION

The proto-oncogene *c-myc* is frequently amplified in many human cancers including breast cancer (1-5), and its amplification is associated with a high proliferation rate (6-7) and poor prognosis (8). Transgenic animal models have confirmed the tumorigenic potential of Myc overexpression in mammary tissue (9-13). However, the mechanism by which Myc affects cell growth has not been elucidated, despite its identification as a transcription factor. In normal cells, *myc* expression is tightly regulated and is correlated with the proliferative state of cell. As a member of the class of “early genes” whose expression is induced a few hours after mitogen stimulation of quiescent cells, Myc is thought to play a critical role in growth regulation (reviewed in 14-16). Since Myc induction if sufficient to drive quiescent cells into the cell cycle (17) and constitutive expression of *c-myc* prevents growth arrest of serum-deprived fibroblasts (18), it has been proposed that *c-myc* controls the expression of genes that are important for cell cycle initiation and/or progression.

The cyclin genes are candidate targets for that proposed action of Myc, since their protein products have been identified as key players in cell cycle regulation (19). It was recently reported that overexpression of *c-myc* in fibroblasts led to deregulated cyclin expression (20-21). We therefore hypothesized that *myc* amplification could function similarly in breast cancer cells, conferring a growth advantage on cells which harbor such a genetic alteration. Aberrant expression of the cyclin genes may contribute to a malignant phenotype since amplifications or gene rearrangements leading to an increase in cyclin A, D1, or D2 mRNA have been found in a number of tumors, including breast cancer (19, 22-23). Several human breast cancer cell lines also overexpress mRNA and protein of cyclins A, B, E and D1(23-24).

However *myc* overexpression can also lead to cell death via apoptosis (18, 25-.27) When *myc*-overexpressing cells are placed under conditions which are normally inappropriate for growth, apoptosis ensues. Thus, it seems likely that cells which overexpress Myc must somehow bypass the apoptotic pathway in order to gain a growth advantage over normal cells. This could

be accomplished in two ways. First, the cells could alter the growth environment by changing growth factor production, in either a paracrine or autocrine fashion. Alternatively, additional genetic changes could selectively block propagation of the apoptotic signal.

The experiments described here were intended to identify components of the pathways through which c-myc acts to both increase proliferation and induce apoptosis in mammary epithelial cells. The studies have focused primarily on two mammary epithelial cell lines (MECs): 1). A1N4-myc cells are chemically immortalized human mammary epithelial cells which demonstrate an accelerated growth rate compared to parental cells but do not undergo apoptosis. 2). Myc 83 cells were derived from a mammary tumor of a myc transgenic mouse and can be induced to undergo apoptosis by altering their growth environment (i.e., removal of EGF or addition of TGF β). By using these two cell lines, both aspects of myc overexpression can be examined. Experiments thus far have focused on changes in mRNA expression (examined via RNase protection assays or Northerns) in proliferating or apoptotic cells (as determined by growth assays, FACS analysis, and various apoptosis assays).

BODY

MATERIALS AND METHODS

Cell lines

The benzo(a)pyrene-immortalized and transformed human mammary epithelial cell line 184A1N4-myc (28) and its parental cell line 184A1N4 were used to study the effects of *myc* overexpression on cell cycle regulation. The A1N4-myc line was established via retroviral infection with a construct containing mouse *myc* under the control of the Moloney mouse leukemia virus long terminal repeat (MMLV LTR). Both cells lines were maintained in media containing 0.5% fetal calf serum (FCS), 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, and 10 ng/ml epidermal growth factor (EGF, Upstate Biotechnology Incorporated, Lake Placid NY). The cells arrest in G0 in the absence of EGF (29).

The myc 83 cell line was established from a mammary tumor of a transgenic mouse (MMTV-c-myc M, 12). In that cell line, *myc* expression is driven by the mouse mammary tumor virus promoter. The cells were routinely grown in media containing 2.5% FCS, 10 ng/ml /EGF and 5 mg/ml insulin. Myc 83 cells undergo apoptosis when EGF is removed from their culture media.

Two pairs of mouse mammary cell lines (MMEC and MMEC-myc, HC14 and HC14-myc) were also used in a number of preliminary experiments. The MMEC line was derived from an 8 week old virgin mammary gland, while HC14 was established from a mid-pregnant gland. In both cases, *myc* expression was driven by the MMLV LTR (30-31).

Anchorage-dependent growth assays

Cells were plated in 96-well plates at a density of 1000-2000 cells/well. When transforming growth factor β (TGFβ, R & D Systems, Minneapolis, MN) was used as a treatment, the media was changed every other day. At various time points, plates were stained with crystal violet (0.5% in 30% MeOH), rinsed with water and dried. At the end of the

experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc.).

FACS Analysis

Cells (A1N4 and A1N4-myc) were plated (5×10^5 cells/plate) in 10 cm dishes (Falcon® 3003, VWR Scientific, Philadelphia, PA) and growth arrested as described above. Following restimulation with EGF (10 ng/ml), cells were harvested at 3 h intervals and nuclei were stained with propidium iodide for cell cycle analysis according to the method of Vindelov et al. (32). Myc 83 cells were plated similarly and then were deprived of EGF for various times before harvesting the cells for FACS.

RNase Protection Assay

Whenever possible, nonradioactive methods were used in place of traditional methods using 32 P. For human cell lines, changes in cyclin RNA expression were examined using a nonradioactive RNase protection assay which we have developed, as described below (33, see appendix A).

RNA Isolation

A1N4 and A1N4-myc cells were plated sparsely (1.5×10^6 cells) in culture flasks (225 cm²; Costar, Cambridge, MA) in normal growth media containing EGF. The next day the cells were changed to EGF-free media to arrest them in G₀. After 48 hours, the cells were restimulated with EGF (10 ng/ml) and RNA was harvested at three hour intervals according to the method of Chomzynski and Sacchi (34). The RNA samples were dissolved in 200 μ l diethyl pyrocarbonate (DEPC)-treated water, aliquoted, and stored as precipitates at -20 °C.

Probe Synthesis

A pGEM-4z vector containing a 400 bp fragment of the human cyclin A cDNA (35) was linearized with EcoRI prior to synthesis of a 440 bp riboprobe. A 1.3 kb NotI fragment of human cyclin D1 (36) in a Bluescript KS- plasmid was linearized with EcoNI to synthesize a 360 bp probe. A 290 bp control probe was produced from an EcoRI-linearized pGEM-4 vector

containing a 220 bp Pst-1 fragment of 36B4, a constitutively expressed ribosomal protein which is frequently used as a control for transcription studies in breast cancer cell lines (37). All riboprobes were synthesized from the T7 promoter.

Probes were synthesized with the Riboprobe II Core System (Promega, Madison, WI). The reactions contained 2 mM biotin-14-CTP (Gibco BRL, Gaithersburg, MD), 1 µg linearized template, 10 µM DTT, 1 mM ATP, CTP, UTP and GTP, 30 U RNasin, and 20 U T7 polymerase in a total volume of 20 µl. After a one hour incubation at 37 °C, the DNA was digested with 3 units of RQI DNase for 15 minutes at 37 °C. The probes were then diluted with DEPC treated water, aliquoted and stored at -70 °C without further purification. The relative efficiency of label incorporation in the various probes was determined by dot blots. Between 1% and 0.1% of the total reaction was used per hybridization.

RPA

The target RNA (60 µg) and probes were co-precipitated with ammonium acetate (0.5 M) and ethanol. The dried pellets were resuspended in 30 µl of hybridization solution (80% formamide, 400 mM NaCl, 1 mM EDTA, 40 mM Pipes, pH 7.0), heated to 85 °C for five minutes, and transferred immediately to a 45 °C water bath. After a 16-hour incubation, the single stranded RNA was digested for 1.5 hours at 37 °C with RNase T1/T2 in 350 µl TNE (0.3 M NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5). The RNase was isolated from *Aspergillus oryzae* (Sigma Chemical Company, St. Louis MO) according to the method of Lichtler and co-workers (1992). The RNases were then inactivated by addition of SDS (0.5%) and proteinase K (0.25 mg/ml, Boehringer Mannheim, Indianapolis, IN). Following a 30 minute incubation at 37 °C, the RNA was extracted with phenol/chloroform, ethanol precipitated, and resuspended in 5 µl loading buffer (80% formamide, 1x TBE, 0.005% xylene cyanol and bromphenol blue).

Samples were heated to 85 °C for 5 minutes and placed on ice prior to loading on a 6% acrylamide gel (Sequagel-6, National Diagnostics, Atlanta, Georgia). The gel (14 x 16 cm x 1 mm) was run in 1x TBE (90 mM boric acid, 1 mM EDTA, 100 mM Tris, pH 8.3) in a Hoefer gel

apparatus (San Francisco, CA) for 1.5 -2 hours at 275 volts. The RNA was then electrophoretically transferred to a charged nylon membrane (Hybond N+; Amersham Life Science) in a Semiphor transfer unit (Hoefer) with 0.2x TBE for 45 minutes at 100 mAmps.

Chemiluminescent Detection

The RNA was UV-crosslinked to the wet membrane after the transfer. Blots were then incubated for 45 minutes at 37 °C in a blocking solution containing 0.5 % SDS and 0.5% blocking agent (Kodak, New Haven, CT) in 100 mM Tris buffered saline (TBS, 100 mM Tris, pH 7.4, 150 mM NaCl). The membrane was incubated 30 minutes at room temperature with streptavidin-horseradish peroxidase (SA-HRP, Kodak) diluted 1/5,000 in blocking solution. Washes consisted of several quick rinses in TBS with 0.1% Tween-20 (TBST) followed by one 15 minute and one 5 minute wash in TBST and then two final 5 minute washes in TBS. The membrane was then incubated with HRP substrate (ECL, Amersham) for 1 minute, placed in a sheet protector, and exposed to film for 5-15 minutes.

Northern Analysis

Since all of our cyclin cDNA probes were human, RNA from the Myc 83 mouse cell line had to be analyzed by standard Northern blots using ³²P labeled probes. Fifteen µg total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL) and hybridized with ³²P-labeled probes generated by a random primed DNA labeling kit (Boehringer Mannheim). For cyclin A, a 1 kb Afl II fragment was labeled; for cyclin D1, a 1.3 kb Not I fragment was used.

Densitometry

The integrated optical density of RNA bands was obtained using the ImageQuaNT image analysis program (Molecular Dynamics, Sunnyvale CA).

Cell Death ELISA

Myc 83 cells were plated in 6-well plates (50,000 cells/well) in normal growth media. The next day, TGFβ was added to the media or the cells were switched to media without EGF.

Twenty-four hours later, the cells were harvested by trypsinization and centrifugation. The cell pellets were resuspended in lysis buffer and kept on ice for 30 minutes. The nuclei were removed by a 10 minute centrifugation and cytoplasmic lysates were stored at -70 °C.

Cytoplasmic apoptotic DNA fragments were detected using an ELISA kit (Boehringer Mannheim) with antibodies directed against histones and DNA. The 96-well plate was coated overnight (4 °C) with the first antibody (anti-histone) and then incubated with lysis buffer for 30 minutes at room temperature (RT). The wells were washed 3 times and then incubated with 100 µl cytoplasmic lysate for 90 minutes (RT). The wells were washed again 3 times and then incubated with the peroxidase-linked second antibody (anti-DNA). Following the final wash, ABTS peroxidase substrate was added. After a 10 minute incubation, color development was detected by measuring absorbance at 410 nm.

RESULTS

In both mouse (HC-14-myc and MMEC-myc) and human (A1N4-myc) MECs, *myc* overexpression decreased the doubling time by about 6 h compared to parental lines (Figure 1, Table 1). Experiments with the A1N4 lines suggested that the difference was not due to increased sensitivity to EGF (Figure 2), but rather to a shortening of G1. A1N4 and A1N4-myc cells were arrested in G₀ in the absence of EGF and were allowed to re-enter the cell cycle by replacing EGF. FACS analysis demonstrated that the A1N4-myc cells began to enter S phase 12 h after EGF addition and percent cells in S phase peaked at 18 h (Figure 3). In contrast, parental cells did not enter S phase until 18 h and peaked at 24 h.

Cell Line	Doubling Time	Difference
A1N4	27.4 h	
A1N4-myc	21.5 h	5.9 h
HC14	25.1 h	
HC14-myc	18.8 h	6.3 h
MMEC ¹	24.0 h	
MMEC-myc ¹	18.2 h	5.8 h

Table 1: Doubling times for 1 human pair and 2 mouse pairs of cell lines (pair=*myc* overexpressing line and its parental line). The last column indicates the decrease in doubling time of the *myc* line compared to its parental line. ¹Telang et al., 1990 (ref. #31).

The shortened G1 phase does not appear to be due to any gross changes in cyclin expression as assessed by a non-radioactive Rnase protection assay which we have developed (33). In unsynchronized cells, *myc* overexpression had no significant effect on cyclin A or D1 mRNA expression. Cyclin mRNA was nearly undetectable in arrested cells and induction was closely correlated with changes in cell cycle phase (Figure 4). In both cell lines, cyclin D1 expression was detectable by three hours after EGF treatment and levels remained relatively constant throughout the cell cycle. A1N4-myc cells began to express cyclin A about 9 hours after EGF stimulation, with a peak at 18 hours. The parental cells showed a delay in cyclin A

expression which is reflected by the FACS data. Our results are in contrast to recent reports that *myc*-overexpressing fibroblasts exhibited increased cyclin A and decreased cyclin D1 expression compared to parental cells in low serum concentration (20-21). This difference may result from the inability of A1N4-myc cells to undergo apoptosis; their growth arrest in the absence of EGF is fully reversible.

We have therefore also examined cyclin expression in the Myc 83 cells which do undergo apoptosis in the absence of EGF. Cyclin A and D1 mRNA levels did not decrease in EGF-deprived Myc 83 cells compared to cells grown in the presence of EGF (not shown). FACS analysis revealed that the cells did not arrest in G1 following removal of EGF (Figure 5), but growth assays clearly demonstrated that the cells were dependent on EGF for growth (Figure 6). Therefore, we suggest that the Myc 83 cells continue to traverse the cell cycle in the absence of EGF, but they undergo apoptosis rather than cell division under those conditions. The effects of c-myc overexpression on cyclin expression would thus appear to be indirect.

Growth assays showed that Myc 83 cells were also extremely sensitive to TGF β , suggesting that the growth factor might be inducing apoptosis rather than G1 arrest, analogous to the removal of EGF. The cell death ELISA demonstrated a strong concentration dependent apoptotic response to TGF β 24 hours after treatment (Figure 7). Microscopic examination of cells stained with Hoechst dye also demonstrated that the cells were undergoing morphological changes which are characteristic of apoptotic cells. Preliminary results suggest that the time course for TGF β -induced apoptosis is quite similar to that observed when EGF is withdrawn.

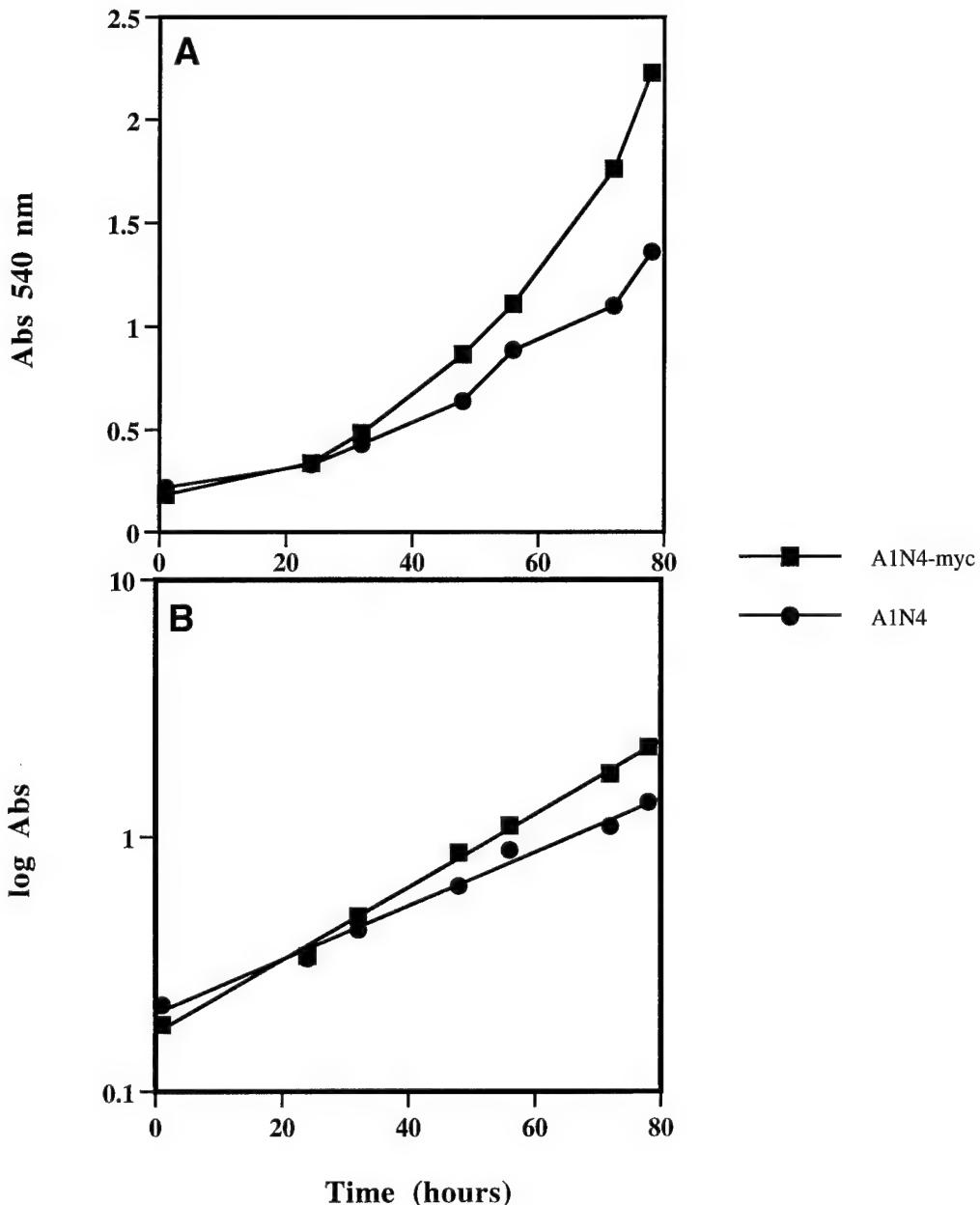


Figure 1: Comparison of the growth rates of A1N4 and A1N4-myc. Cells were seeded in 96-well plates at a density of 1500 cells/well in normal growth media. At the times indicated, relative cell number was determined via crystal violet staining (A). In panel B, the log of the absorbance values in A were plotted against time and the slopes of the resultant lines were used to calculate doubling time (DT). For A1N4, DT=27.4 h, +/-0.6; for A1N4-myc DT=21.5, +/-0.3.

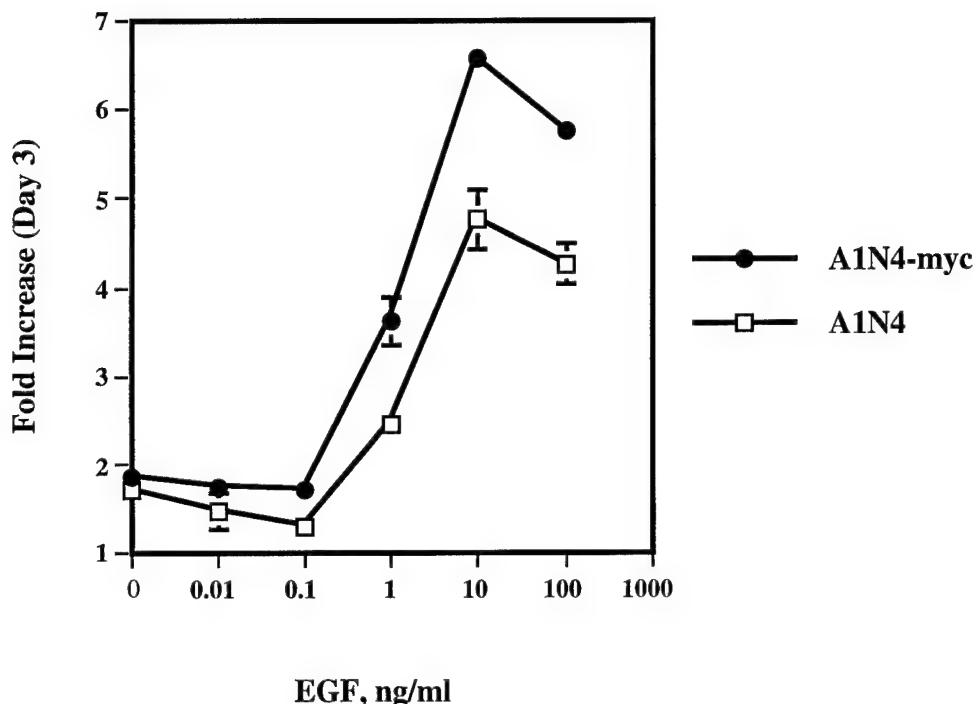


Figure 2: Growth of A1N4 and A1N4-myc cells in increasing concentrations of EGF. Cells were seeded in 96-well plates at a density of 1500 cells/well in normal growth media with varying concentrations of EGF. On day three, relative cells number was determined via crystal violet staining. At each concentration, A1N4-myc cells grew faster than parental cells.

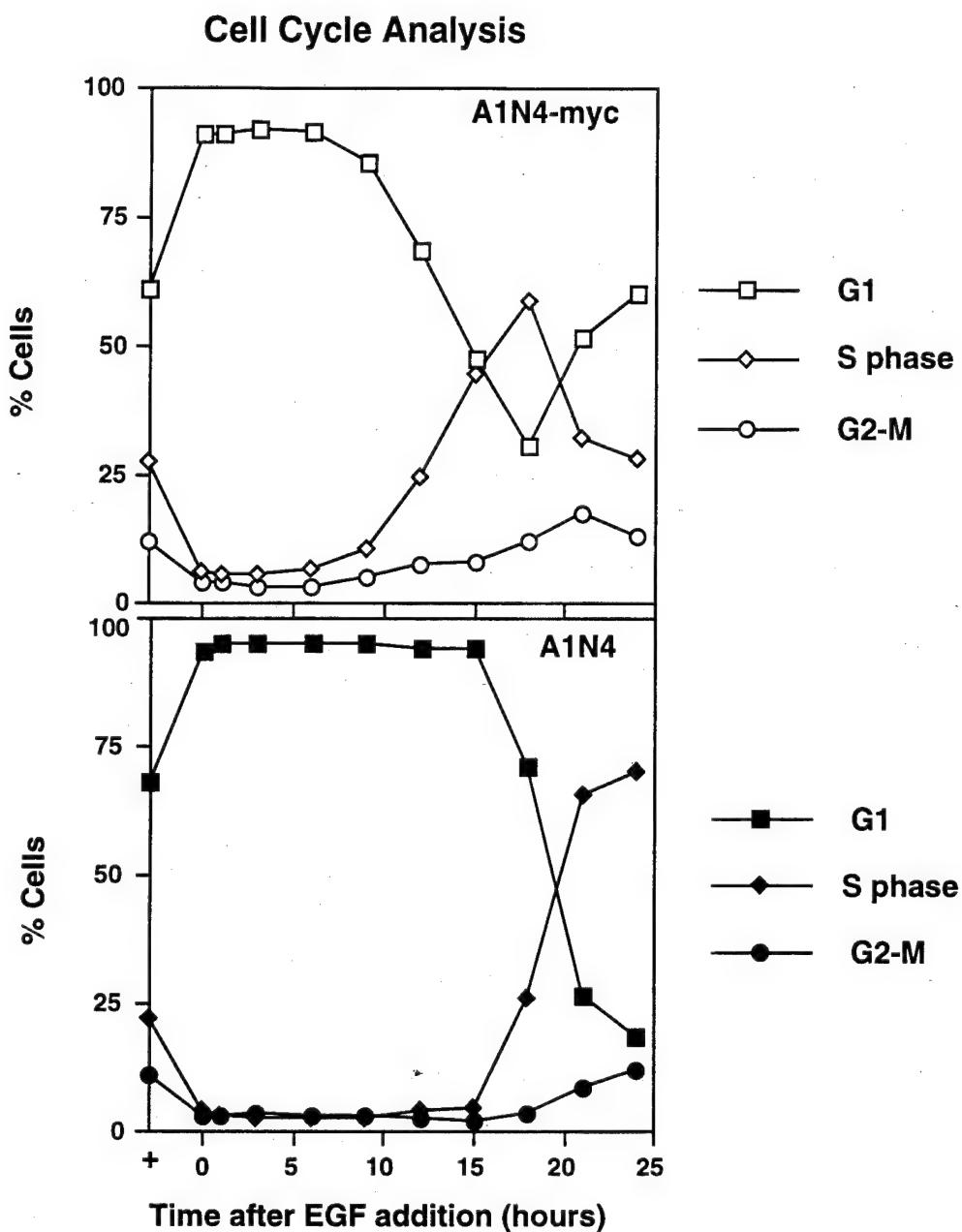


Figure 3: Cell cycle analysis of A1N4 and A1N4-myc cells re-stimulated with EGF following growth arrest. Arrested cells were treated with 10 ng/ml EGF and harvested at 3 hour intervals. Staining and FACS analysis of the cells was performed as described in Materials and Methods. + = unsynchronized cells.

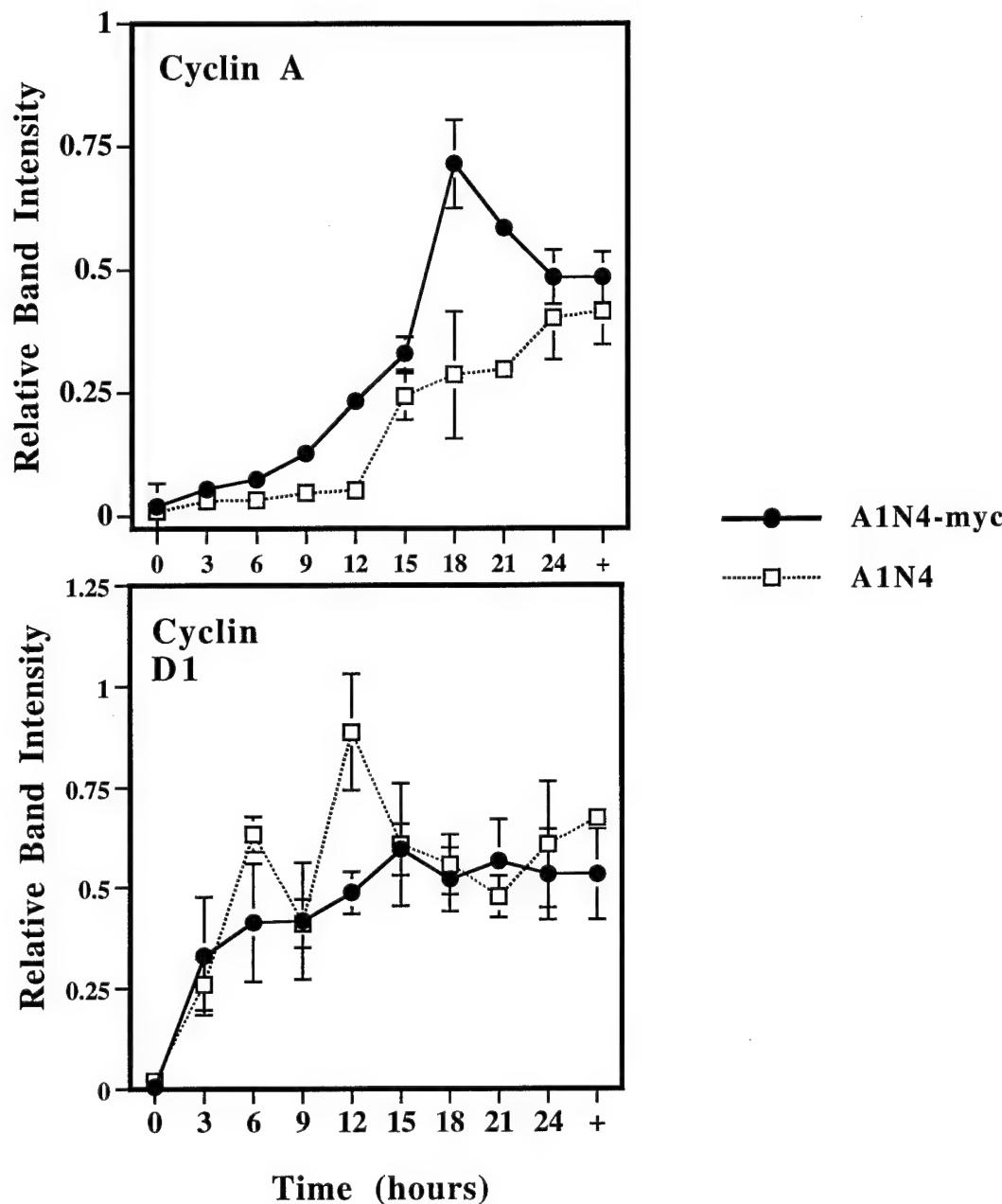


Figure 4: Cell cycle dependent expression of cyclin D1 and cyclin A in A1N4 and A1N4-myc cells. Cells were arrested in G0 by EGF deprivation and re-stimulated by addition of EGF. At the times indicated, total RNA was harvested and analyzed by RNase protection assays. Densitometric measurements of cyclin RNA bands were normalized by the relative density of the control 36B4 bands.

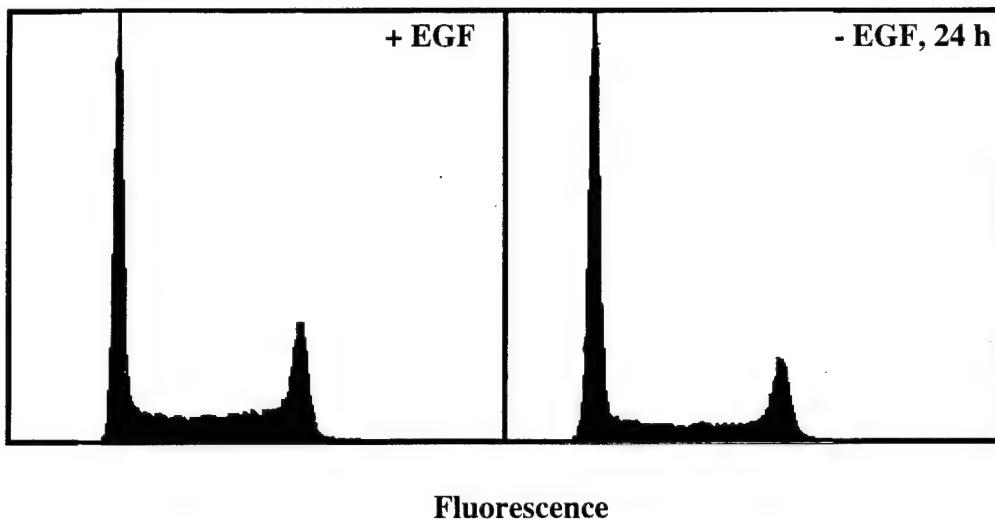


Figure 5: Myc 83 cells do not arrest in G0/G1 in the absence of EGF.
 Cells were grown in normal media with or without EGF for 24 h.
 Nuclei were then isolated and subjected to FACS cell cycle analysis.

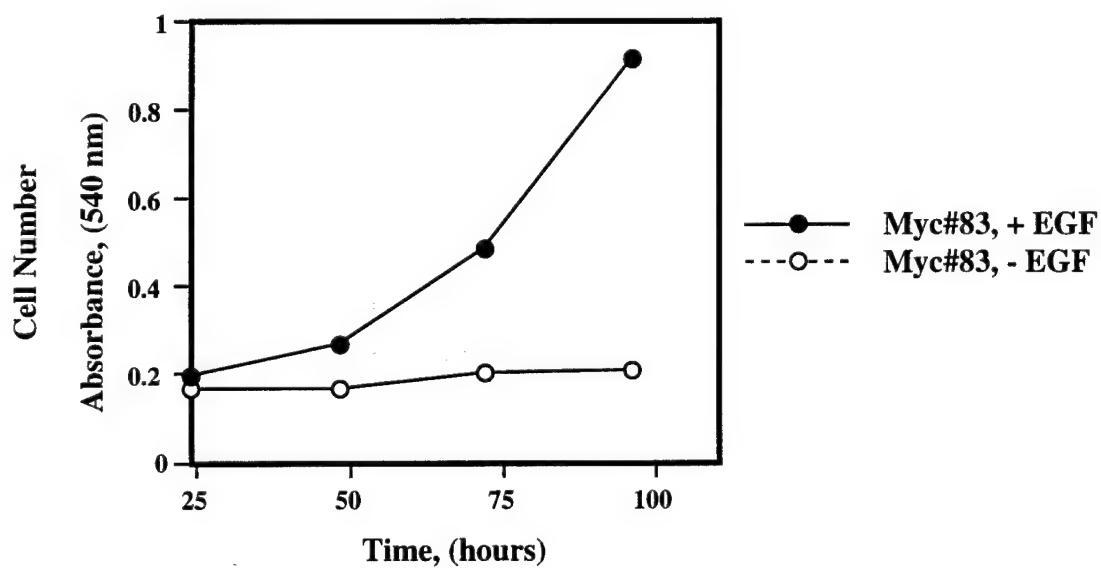


Figure 6: Myc83 cells are dependent on EGF for proliferation.
 Cells were seeded in 96-well plates at a density of 1500 cells/well
 and were grown in the presence or absence of EGF. At the indicated
 times, plates were stained with crystal violet.

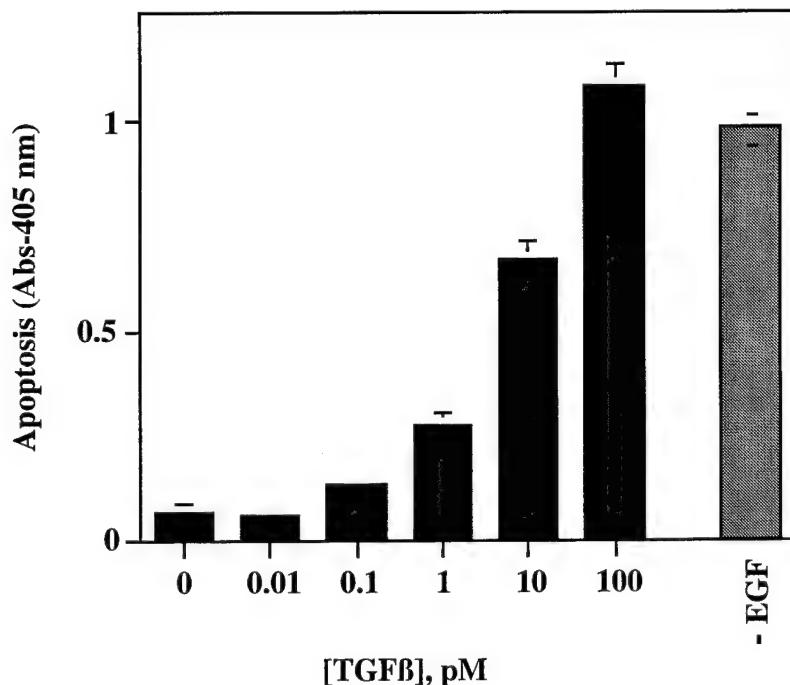


Figure 7: TGF β induces apoptosis in Myc 83 cells.

Cells were seeded in 6-well plates at a density of 50,000 cells/well.

The next day, various concentrations of TGF β were added. 24 hours later, cytoplasmic lysates were prepared and relative increases in apoptosis were measured with the Cell Death ELISA kit. Withdrawal of EGF for 24 hours was used as a positive control.

CONCLUSIONS

Our results for SOW task 1 indicate that the effects of c-myc on cyclin expression are indirect and simply reflect the cell cycle status of the cells. We therefore suggest that our original hypothesis has been disproved and that tasks 2 and 3 are no longer the most logical directions to proceed. Thus alternative experiments will be undertaken as described below.

We have demonstrated that c-myc can accelerate cell growth or induce apoptosis, depending on the cell line and the cell environment. The presence or absence of inhibitory or stimulatory growth factors such as TGF β and EGF can determine the fate of cells which overexpress c-myc (Figure 8). However, the pathways through which c-myc and the growth factors act to stimulate growth or apoptosis in MECs are not known. The experiments below will begin to address that question.

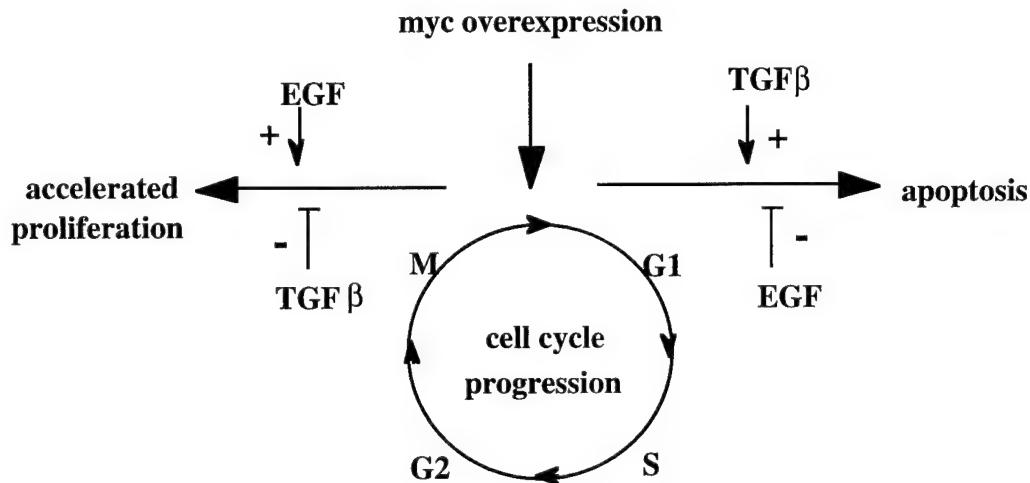


Figure 8: Proposed model for the effects of c-myc overexpression on mammary epithelial cells. A1N4 and A1N4-myc cells have been used to study the left side of the model (accelerated proliferation) while Myc 83 cells will be used to investigate the induction of apoptosis (right side).

The first set of experiments will examine expression of the apoptosis-related genes Bax, Bcl-2, and Bcl-X in Myc 83 cells by Northern and Western analysis. The cells will be plated in

normal growth media with EGF. The next day, the media will be replaced with the following treatments: 1) + EGF, 2) - EGF, 3) + EGF +TGF β . Cell lysates will be prepared and total RNA will be harvested from the cells 24 hours later.

Using similar treatments and analysis, expression of p53 will also be assessed. For the Western blots, several antibodies which specifically recognize the different conformational states of p53 will be used. Immunohistochemistry will also determine the cellular location of p53, since it can be sequestered in the cytoplasm. The latter 2 experiments will begin to assess p53 activity as well as relative protein levels.

We will also continue the analysis of A1N4 and A1N4-myc cells. As stated in the results, the decreased doubling time of A1N4-myc cells appears to be due to a reduction in the length of G1. Thus, studies in this cell line will concentrate on the regulation of passage through G1 and the transition into S phase. Since expression of cyclins D1 and A was not grossly altered compared to parental cells, future studies will focus on the activity of cyclin-cdk complexes. We hypothesize that constitutive c-myc might lead to reduced expression or activity of the cdk inhibitors, accelerating Rb-1 (retinoblastoma protein) phosphorylation, and thereby reducing time spent in G1. The first experiment will determine the time course of Rb-1 phosphorylation since Rb-1 is a primary target of the G1 cdk's. A1N4-myc cells will be arrested in G1 by EGF withdrawal and then restimulated. At various times after EGF addition, cell lysates will be harvested and analyzed on a western blot with antibodies directed against Rb-1. By running the proteins on a 6% gel, the hypo- and hyper-phosphorylated forms of Rb-1 can be resolved into distinct bands. If there is a dramatic difference in the onset of Rb-1 phosphorylation, additional experiments will determine the time course of cdk 4 activation. Using a cyclin-dependent kinase assay kit from UBI (Lake Placid, New York), cdk4 will be immunoprecipitated from the lysates and the kinase activity will be assessed. The immunoprecipitates will also be run on a gel to determine whether they are associated with cdk inhibitors (by Western blots).

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APPENDIX

Nass S.J. and Dickson R.B. Detection of cyclin messenger RNAs by nonradioactive ribonuclease protection assay: A comparison of four detection methods. BioTechniques (In press, scheduled for publication in November, 1995).

Short Technical Reports

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SHORT TECHNICAL REPORTS

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Detection of Cyclin Messenger RNAs by Nonradioactive Ribonuclease Protection Assay: A Comparison of Four Detection Methods

BioTechniques 19: ____ (October 1995)

ABSTRACT

Stable, nonradioactive riboprobes were used in ribonuclease protection assays to monitor the changes in cyclin mRNA expression during cell cycle progression in human mammary epithelial cells. Probes labeled with biotin demonstrated sufficient sensitivity (comparable to ^{32}P) to detect these low-abundance mRNAs and thus offer a safe and easy alternative to traditional radioactive assays. Three detection systems based on chemiluminescence generated by horseradish peroxidase or alkaline phosphatase were compared for sensitivity, background and ease of use.

INTRODUCTION

In recent years, there has been considerable pressure to eliminate the use of radioactive isotopes in biological research. For some techniques, this transition has been made quite successfully. However, the poor sensitivity of the published methods for nonradioactive Northern analysis precludes their use for the detection of low-abundance mRNAs. A nonradioactive ribonuclease protection assay (RPA) is a logical alternative to nonradioactive Northerns. RPA sensitivity is at least an order of magnitude higher than that of Northern blots, in part because electrophoretic separation of the protected probe from the degraded, unhybridized probe reduces background and partly because the amount of target RNA can be dramatically increased (up to 80 μg in the former vs. 20 μg in the latter method).

One method for nonradioactive RPA using biotinylated probes was recently reported (8). In that protocol, a biotinylated probe was detected by strepta-

vidin-linked alkaline phosphatase and a chemiluminescent substrate. However, in our laboratory, the method had an unsatisfactory signal-to-background ratio for detection of the human cyclin mRNAs. We have therefore established methods using streptavidin-linked peroxidase detection.

MATERIALS AND METHODS

RNA Isolation

The immortalized and transformed human mammary epithelial cell line 184A1N4-myc (9) was plated sparsely (1.5×10^6 cells) in culture flasks (225 cm^2 ; Corning Costar, Cambridge, MA, USA) in its normal growth media containing epidermal growth factor (EGF). The next day the cells were changed to EGF-free media to arrest them in G_0 (7). After 48 h, the cells were re-stimulated with EGF (10 ng/mL; Upstate Biotechnology, Lake Placid, NY, USA) and RNA was harvested at 3-h intervals according to the method of Chomzynski and Sacchi (1). The RNA samples were dissolved in 200 μL diethyl pyrocarbonate (DEPC)-treated water, aliquoted and stored as precipitates at -20°C.

Probe Synthesis

A pGEM®-4Z vector (Promega, Madison, WI, USA) containing a 400-bp fragment of the human cyclin A cDNA (6) was linearized with *Eco*RI prior to synthesis of a 440-bp antisense riboprobe. A 290-bp control probe was produced from an *Eco*RI-linearized pGEM-4Z vector containing a 220-bp *Pst*I fragment of 36B4, a constitutively expressed ribosomal protein that is frequently used as a control for transcription studies in breast cancer cell lines (3). Both riboprobes were synthesized from the T7 promoter. To test the sensitivity of the methods, an unlabeled cyclin A sense riboprobe was also synthesized from the SP6 promoter following linearization of the vector with *Bst*YI.

Radioactive probes were synthesized with the Riboprobe® II Core System (Promega) on the same day as solution hybridizations were begun. The reactions contained 50 μCi [α - ^{32}P]UTP (3000 Ci/mmol, 10 mCi/mL; Amer-

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sham, Arlington Heights, IL, USA), 1 μ g linearized template, 10 μ M dithiothreitol (DTT), 0.375 mM ATP, CTP and GTP, 10 μ M unlabeled UTP, 30 U RNasin[®] and 20 U T7 polymerase in a total volume of 20 μ L. After a 1-h incubation at 37°C, the DNA was digested with 3 U of RQI DNase for 15 min at 37°C. The probe was then extracted with phenol/chloroform, ethanol-precipitated and resuspended in 200 μ L of DEPC-treated water. Fifty thousand dpm of each probe were used per hybridization.

The biotinylated probes were synthesized in a similar reaction with the following modifications. The labeled nucleotide consisted of biotin-14-CTP (Life Technologies, Gaithersburg, MD, USA) at a concentration of 2 mM. All four unlabeled nucleotides were present at a concentration of 1 mM. After the DNase digestion, the probes were diluted with DEPC-treated water, aliquoted and stored at -70°C without further purification. The relative efficiency of label incorporation in the various probes was determined by dot blots (not shown). Between 1% and 0.1% of the total reaction was used per hybridization.

RNase Protection Assay

The target RNA (60 μ g) and probes were co-precipitated with ammonium acetate (0.5 M) and ethanol. The dried pellets were resuspended in 30 μ L of hybridization solution (80% formamide, 400 mM NaCl, 1 mM EDTA, 40 mM PIPES, pH 7.0), heated to 85°C for 5 min and transferred immediately to a 45°C water bath. After a 16-h incubation, the single-stranded RNA was digested for 1.5 h at 37°C with approximately 50 U of RNase T1/T2 in 350 μ L TNE (0.3 M NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5). The RNase was isolated from *Aspergillus oryzae* (Sigma Chemical, St. Louis, MO, USA) according to the method of Lichtler and co-workers (4) and activity was determined by a standard method (2). The RNases were then inactivated by addition of sodium dodecyl sulfate (SDS) (0.5%) and proteinase K (0.25 mg/mL; Boehringer Mannheim, Indianapolis, IN, USA). Following a 30-min incubation at 37°C, the RNA was extracted with phenol/chloroform, ethanol-pre-

cipitated and resuspended in 5 μ L loading buffer (80% formamide, 1× TBE [90 mM boric acid, 1 mM EDTA, 100 mM Tris, pH 8.3], 0.005% xylene cyanol and bromophenol blue).

Samples were heated to 85°C for 5 min and placed on ice prior to loading on a 6% acrylamide gel (SequagelTM; National Diagnostics, Atlanta, GA, USA). The gel (14 × 16 cm × 1 mm) was run in 1× TBE in a Hoefer gel apparatus (Hoefer Pharmacia Biotech, San Francisco, CA, USA) for 1.5–2 h at 275 V. The RNA was then electrophoretically transferred to a charged nylon membrane (HybondTM-N+; Amersham) in a SemiPhorTM transfer unit (Hoefer Scientific) with 0.2× TBE for 45 min at 100 mA. Blots of the ³²P-labeled probes were covered with plastic wrap and first exposed to Fuji RX x-ray film (Fuji Medical Systems, Stamford, CT, USA) for 17 h at -70°C with an intensifying screen (Du Pont Cronex[®] Quanta III, Sigma Chemical). The blot was then also analyzed with a phosphorimager (see below). Autoradiography of the gel demonstrated that transfer was complete under these conditions.

Chemiluminescent Detection

The RNA was UV-cross-linked to the wet membrane after the transfer. All blots were then incubated for 45 min at 37°C in a blocking solution containing 0.5% SDS and 0.5% blocking agent (IBI/Kodak, New Haven, CT, USA) in 100 mM Tris-buffered saline (TBS; 100 mM Tris, pH 7.4, 150 mM NaCl). A variety of blockers were examined (including bovine serum albumin [BSA] and casein) and the IBI/Kodak blocker was chosen because it provided the highest ratio of sensitivity to background (not shown). Two detection methods utilizing streptavidin-horse-radish peroxidase (SA-HRP, IBI/Kodak) were tested. For both, the membrane was incubated 30 min at room temperature with SA-HRP diluted 1/10000 in blocking solution. Washes consisted of several quick rinses in TBS with 0.1% Tween 20 (TBST), followed by one 15-min and one 5-min wash in TBST and then two final 5-min washes in TBS. For the first method, the membrane was simply incubated in a liquid HRP substrate (ECL[™], Amersham) for 1 min, placed in a sheet pro-

tection and exposed to film for 5 min. The blot was then rinsed for 15 min in TBS prior to testing the second method on the same membrane. For that method, the blot was rinsed in equilibration buffer (IBI/Kodak), then overlaid with a solid HRP substrate matrix (IBI/Kodak) and exposed to film for 30 min. Previous experiments had shown that the time required for film exposure was not significantly different when applying the substrate matrix before or after short exposures of the blot to ECL. With the IBI/Kodak system, light emission continues for approximately 2 h. Use of the ECL substrate results in light emission for about 1 h with a peak at approximately 20 min.

A second blot was prepared using identical RNA samples and was blocked and washed as described above. The SA-HRP, however, was replaced with streptavidin-alkaline phosphatase (SA-AP; United States Biochemical, Cleveland, OH, USA), and the TBS was autoclaved to eliminate any endogenous alkaline phosphatase activity. After the last wash, the blot was rinsed in AP buffer (100 mM NaCl, 50 mM MgCl₂, 100 mM Tris, pH = 9.5) and then 1 mL of Lumi-Phos[™] 530 (Boehringer Mannheim) was applied. Three hours later, a 1.5-h exposure to film was made. In this system, light emission increases linearly for about 8 h, remains constant for another 12 h and then decreases slowly over the next 48 h.

Densitometry

The integrated optical density of radioactive RNA bands was obtained using the PhosphorImager[™] 445 SI and the ImageQuaNT[™] image analysis program (Molecular Dynamics, Sunnyvale, CA, USA). Chemiluminescent films were analyzed with the Personal Densitometer[™] SI (Molecular Dynamics) and ImageQuaNT.

RESULTS AND DISCUSSION

Cyclins are the regulatory subunits of cyclin-dependent kinases (CDKs), which orchestrate passage through the cell cycle (5). Expression of the various cyclins is tightly regulated and is indicative of specific stages of the cell cy-

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cle. We have developed a nonradioactive RNase protection assay, which can be used to monitor expression of cyclin mRNAs in cultured cells.

Figure 1 compares the results obtained using biotinylated probes with three different detection systems to those obtained using standard radioactive probes. The protected fragments for cyclin A and 36B4 were 400 bp and 220 bp, respectively. However, since biotin significantly increases the apparent molecular weight of the probes, a direct determination of the size of the biotinylated probes cannot be made by comparison to the migration of unlabeled molecular weight standards. The protected fragments can be distinguished from the original probe by running a sample of undigested probe for comparison.

The changes in cyclin A mRNA expression detected by a radioactive probe (Figure 1A) were quite similar to the changes seen using biotinylated probes with two SA-HRP-based detection systems (Figure 1, B and C), but not with an SA-AP-based method (Figure 1D). Furthermore, the time course of cyclin A expression observed using the first three methods (A-C) closely

mirrored the progression of the cells through the cell cycle (not shown), as determined by FACS analysis according to the method of Vindelov et al. (10). Cyclin A mRNA increased dramatically between 9 and 18 h after EGF stimulation, reflecting the corresponding change in the percentage of cells entering S phase. Cyclin A, whose expression is turned on at the G1-S transition, has been implicated in the progress of DNA replication and in mitosis (5). In contrast, the amount of control 36B4 mRNA is essentially constant throughout the time course.

However, for reasons unknown, the intensity of the 36B4 bands was much higher using radioactively labeled probes than with biotinylated probes. Preliminary results indicate a similar phenomenon with a 120-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (not shown). This discrepancy was not due to a low concentration of biotinylated 36B4 probe, since probe titrations were performed at the onset of the study. Optimal probe concentration was chosen so that doubling the probe concentration resulted in increased background without a significant change in protected band inten-

sity, while decreasing the probe concentration to 1/2 or 1/4 did not alter background or protected band intensity. Since 36B4 was only used as a loading control to normalize quantitation of the cyclin bands in this study, the difference in band intensity did not cause a serious problem. When the band density ratios of cyclin A to 36B4 are plotted on the same scale, the pattern is nearly identical for the radioactive and biotinylated probes (Figure 2). Thus relative changes can still be calculated. If a direct quantitative comparison of multiple RNAs was required, these results could be a cause for concern, but such comparisons are not commonly made because it is difficult to account for differences in the specific activity of multiple probes.

Clearly the results obtained with ECL (Figure 1B) and the solid HRP substrate matrix (Figure 1C) are qualitatively and quantitatively (Figure 2) similar. The only real differences between the two detection methods are the length of time required for film exposure and the relative ease of use. Since light emission reaches a maximum more quickly with ECL, multiple exposures can be made in a very short period of time. In addition, the solid matrix was a bit more tedious to use since care must be taken to remove bubbles trapped between the matrix and the blot. Adjustments must also be

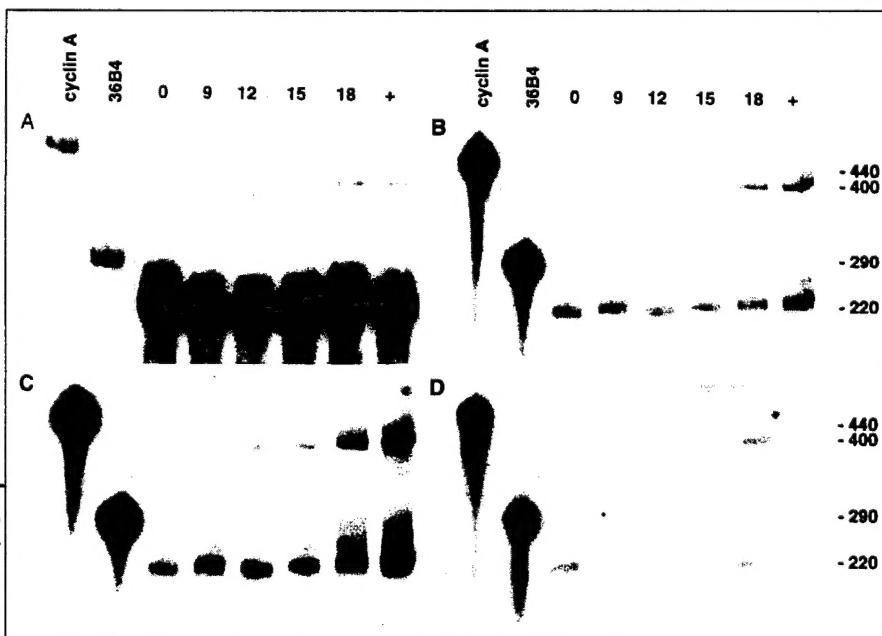


Figure 1. RPA analysis of cyclin A and 36B4 mRNA by four detection methods. Ribonuclease protection assays were performed as in Materials and Methods. A) α -³²P-UTP-labeled probes. The bands in lanes 1 and 2 are from a 2-h exposure; all others, 17 h. B) Biotin-14-CTP labeled probes, SA-HRP, ECL substrate. C) Biotinylated probes, SA-HRP, IBI/Kodak substrate matrix. D) Biotinylated probes, SA-AP, Lumi-Phos 530 substrate. Numbers indicate time after EGF addition; + = unsynchronized cells. Protected cyclin A fragment = 400 bp, protected 36B4 fragment = 220 bp.

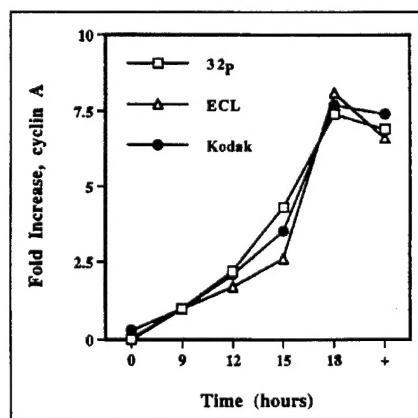


Figure 2. Relative band intensity ratios of cyclin A to 36B4 from RPA using ³²P and biotin probes. PhosphorImager data from the blot in Figure 1A and optical densities of the bands in Figure 1, B and C, and were determined as described in Materials and Methods. The ratio of cyclin A to 36B4 at 9 h was assigned a value of 1, and all other values were calculated as a percent increase over 9 h. + = unsynchronized cells.

made for different-sized blots because the dimensions of the matrix are relatively small (4×5 in.).

Figure 1D shows that the results using biotinylated probes and SA-AP detection are of lesser quality than with radioactive probes or SA-HRP detection. The ratio of band intensity to background was significantly reduced compared with the other three methods shown in Figure 1. A variety of blocking agents and conditions were tested with this detection method, but none were able to reproduce the quality of results obtained with the two SA-HRP-based methods (not shown). SA-AP detection also required significantly longer film exposure times. This was probably due to the slow increase in light emission, which is characteristic of Lumi-Phos 530. We tried incubating the blot in the dark until maximum emission was achieved before starting a film exposure but found that increased background as well as band intensity negated any advantage over earlier exposures. Furthermore, the stronger bands actually decreased in intensity, presumably due to exhaustion of the substrate.

To quantitatively test the sensitivity of our method, RNase protection assays were performed with serial dilutions of an unlabeled sense cyclin A riboprobe mixed with 60 μ g of yeast RNA and 2 μ L of labeled cyclin A antisense probe. As little as 0.1 pg sense RNA could be detected with the biotinylated probe in conjunction with SA-HRP and ECL substrate (not shown). Similar results were obtained with a 32 P-labeled probe after a 3-day exposure to film. However, if the exposure time was extended to 7 days, a faint band could also be detected for 0.01 pg of target RNA.

As previously noted (8), artifactual bands of undigested probe tend to be stronger when using biotinylated probes than with 32 P probes. RNA polymerase occasionally initiates synthesis at the open ends of the plasmid, generating a small amount of full-length sense RNA. That phenomenon would probably be more frequent during the synthesis of biotinylated probes where the reaction contains an excess of all four nucleotides than in a reaction where [32 P]UTP is limiting. Alternatively, biotinylation may render a por-

tion of the RNA insoluble or resistant to nuclease digestion. The intensity of the artifactual bands is dependent upon the amount of probe added and does not reflect the amount of target RNA in the sample. Unprotected probe can be detected even when no target RNA is included in the assay (8) or when the mammalian target RNA is replaced with yeast RNA (not shown).

We chose to use RNase T1/T2 rather than the more common RNase A because Turnbow and Garner (8) reported that use of RNase A (alone or mixed with T1) with biotinylated probes produced broad, fuzzy bands, presumably due to a destabilization of the RNA hybrids. They performed the digestion with RNase T1 (1000 U/mL; Boehringer Mannheim) for 60 min at 30°C. We found that optimal results were obtained using a relatively low RNase concentration (150 U/mL) in a relatively long digestion (1.5 h) at 37°C. This was determined by testing various times, temperatures and concentrations (not shown).

The only inconvenience of using nonradioactive probes for RPA is that direct quantitation of label incorporation cannot be made. We have made relative comparisons of the various probes by dot-blot analysis. Serial dilutions of the probes were spotted onto nylon and then detected by SA-HRP or SA-AP. Probes containing fluorescein, however, could offer the advantage of direct visualization in estimating the efficiency of probe incorporation. A small amount of probe spotted onto a membrane can be visualized on an ultraviolet transilluminator after a brief wash to remove unincorporated nucleotides. In preliminary experiments, we have detected cyclin A mRNA with a fluorescein-labeled probe (not shown).

The primary advantage of using biotinylated probes for RPA is the elimination of radioactivity in the procedure. Use of radioactive materials requires a license, careful monitoring of the work area for contamination and tedious and expensive disposal procedures. The methods described here offer a convenient alternative for laboratories that are unable or unwilling to deal with obstacles involved with radioactive isotope use. In addition, 32 P-labeled nu-

cleotides and riboprobes disintegrate within days of synthesis and must therefore be newly synthesized for each RNase protection assay. In contrast, nonradioactive probes are quite stable. A single labeling reaction can be stored indefinitely at -70°C and can thus be used to analyze hundreds of RNA samples.

The methods described here have broad applications. Specifically, the nonradioactive detection of cyclin mRNAs would be useful for anyone investigating cell cycle regulation. More generally, any laboratory studying gene expression could potentially adapt the techniques for their particular gene of interest, thereby eliminating or at least reducing the use of 32 P.

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Chemiluminescent and Colorimetric Enzymatic Assays for the Detection of PCR-Amplified *Salmonella* sp. Products in Microplates

Just checking correct spelling or Johnson?
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ABSTRACT

To improve Salmonella detection, we developed nonradioactive hybridization assays of amplified products from pure *Salmonella* cultures. Biotin-labeled PCR products were trapped by internal probes covalently bound to CovaLink-NH MicroWells® and detected by colorimetric or chemiluminescent enzymatic reactions. The sensitivities of colorimetric assays using peroxidase and alkaline phosphatase were similar to those obtained with an ethidium bromide-stained agarose gel; both procedures allow the detection of 50 *Salmonella* cells. Chemiluminescence was 10-fold more sensitive than colorimetry.

INTRODUCTION

Since the development of polymerase chain reaction (PCR) amplification (6), a range of PCR-based assays has been developed for rapid screening of pathogenic microorganisms such as *Salmonella* (3,4). PCR results have generally been analyzed by ethidium bromide-stained agarose gels. This detection method is rapid but hazardous because it uses a mutagenic compound. To be widely used in the food industry, *Salmonella* detection techniques must be safe, easy to handle and automated. Hence, we developed hybridization assays of amplified products in a microplate format. An oligonucleotide [ST14 (1)] was immobilized onto CovaLink-NH microplates (Nunc, City _____, Denmark) and used as an internal capture probe for PCR products generated using biotin-labeled primers [ST11 and ST15 (1)]. Amplified biotinylated fragments were detected using an enzyme-streptavidin conjugate.

For colorimetric tests, we investigated chromogenic substrates of peroxidase and alkaline phosphatase. For chemiluminescent assays, we evaluated only one substrate, Lumi-Phos™ 530. After optimization of hybridization conditions, we compared the performance of these three detection methods using pure cultures of *Salmonella*.

MATERIALS AND METHODS

Preparation of Samples for PCR

Samples for PCR were either dilutions of *Salmonella Typhimurium* LT2 DNA that were extracted according to Chen and Kuo's method (2) or dilutions of *S. Typhimurium* LT2 culture that were boiled for 10 min prior to PCR.

PCR Conditions—Agarose Gel Analysis

Ten microliters of sample were added to the reaction mixture (90 µL) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, 0.6 µM of each oligonucleotide primer: biotin-labeled ST11 and ST15 (1), 100 µM of each dNTP. PCR was performed with a Model 9600 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). PCR samples were denatured at 95°C for 10 min, and 1 U of *Taq* DNA Polymerase (Boehringer Mannheim, Meylan, France) was added at 90°C. Next, 35 cycles were run under the following conditions: 30 s at 92°C, 30 s at 60°C and 30 s at 72°C, followed by a final extension for 10 min at 72°C. Ten microliters of amplified products were analyzed by electrophoresis on an ethidium bromide-stained 2% agarose gel.

Immobilization of the Capture Probe

The immobilization method of the internal probe ST14 onto CovaLink-NH (Nunc, Rockville, IL, USA) was previously reported (6). Before use, the MicroWells® were stored at 4°C in 100 µL of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Hybridization Conditions

Amplified products were denatured for 10 min at 100°C and cooled on ice. For hybridization, 10 µL of PCR